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Characterization of histone and protamine variants in sperm of the bivalve mollusc *Aulacomya ater*

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The sperm nucleus of *Aulacomya ater*, family Mytilidae, order Mytiloida, contains three proteins (X, Aa5 and Aa6) which are specific of this cell. These proteins have been isolated by CM chromatography and preparative electrophoresis. Electrophoretic analysis, amino acid composition, M_r determination and tryptic digestion show that protein X corresponds to a histone-like protein whereas Aa5 and Aa6 are protamine-like proteins.

(Sperm) *Chromatin* *Histone-like protein* *Protamine-like protein*

1. INTRODUCTION

Sperm nuclei of Bivalvia mollusca display a high degree of variability among their DNA-associated proteins. In fact, several authors have studied the basic protein content of related sperm cells [1–5] and have pointed out the coexistence of both somatic-type histones and sperm-specific proteins. Some among the latter have been considered intermediate between histones and protamines.

As regards the sperm nucleus of *Aulacomya ater*, its protein complement is made up of the four core histones plus three additional components which are not present in somatic cells of the same organism [5].

Here, we report the isolation, purification and characterization of the three *A. ater* sperm-specific components mentioned (X, Aa5, Aa6). On the basis of the present results, assignment is made of the latter proteins to both histone- and protamine-like variants, respectively. Furthermore, it is worth noting the high degree of homology of these components with others present in spermatozoa of species belonging to the family Mytilidae [1,3,5,17].

2. MATERIALS AND METHODS

2.1. *Isolation of histone and protamine variants*

Specimens of *A. ater* were collected off the Chilean coast, from Puerto Montt Bay and immediately processed. All operations except for chromatographic purification and trypsin digestion were performed at 4°C as described [5].

Nuclei and basic proteins were obtained as in [1]. 0.1 mM phenylmethylsulfonyl fluoride was present as proteolytic inhibitor throughout the procedure.

The acid-soluble protein fraction was stirred overnight in a buffer containing 1 M NaCl, 50 mM sodium acetate, 0.02% sodium azide, pH 6.7, and thereafter centrifuged at 3500 × *g* for 15 min. The supernatant was loaded onto a carboxymethyl-Sephadex C25 column equilibrated with the above buffer and eluted with 1–2 M NaCl linear gradient made up in the acetate buffer as shown in fig.1. Protein Aa5 was obtained in a pure form (peak 3) whereas the remaining two components (X, Aa6) co-eluted together in peak 2.

Purification of proteins X and Aa6 was resolved by preparative electrophoresis on acetic acid-urea

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slab gels containing 15% acrylamide (w/v) followed by electroelution as described [6].

2.2. *Gel electrophoresis*

Proteins were analyzed by polyacrylamide gel electrophoresis as in [7,8]. Both acetic acid-urea and SDS gels were stained with Coomassie blue R250 in 25% methanol and 7% acetic acid. Acetic acid-urea gels for basic peptides were stained as in [9].

2.3. *Amino acid analysis*

Protein samples were hydrolyzed under vacuum in 6 M HCl for 24 h. Amino acid analyses were performed on a Beckman 119CL amino acid analyzer. Corrections were made for hydrolytic losses.

2.4. *Trypsin digestion*

Proteins X and Aa5 were digested with trypsin at room temperature in 20 mM Tris-HCl, pH 8.0, 1.5 M NaCl using an enzyme:substrate ratio of 1:1000 (w/w). Digestion was stopped by addition of trypsin inhibitor-agarose at a ratio of enzyme:inhibitor of 1:4 (w/w) followed by precipitation with 25% trichloroacetic acid. Samples were centrifuged in an Eppendorf microfuge for 10 min, successively washed with acetone-HCl and acetone and finally dried under vacuum. Samples for electrophoresis were dissolved in 8 M urea, 5% β-mercaptoethanol and 5% acetic acid.

3. RESULTS AND DISCUSSION

Acid-soluble proteins were extracted from whole male gonads as described in section 2. The basic protein complement consists of a mixture of histones and sperm-specific proteins (fig.2, lanes 1). Protein Aa5 was resolved from the other proteins by chromatography on CM-Sephadex C-25 as shown in fig.1. Proteins X and Aa6 co-eluted together in peak 2 at about 1.18 M NaCl.

Proteins X and Aa6 could be separated by preparative electrophoresis as described in section 2. Proteins X, Aa5 and Aa6 appeared to be in a pure form as assessed by polyacrylamide gel electrophoresis both in acetic acid-urea gels (fig.2A, lanes 2–4) and acrylamide-SDS (fig.2B, lanes 2–4). On acetic acid-urea gels, protein X runs in

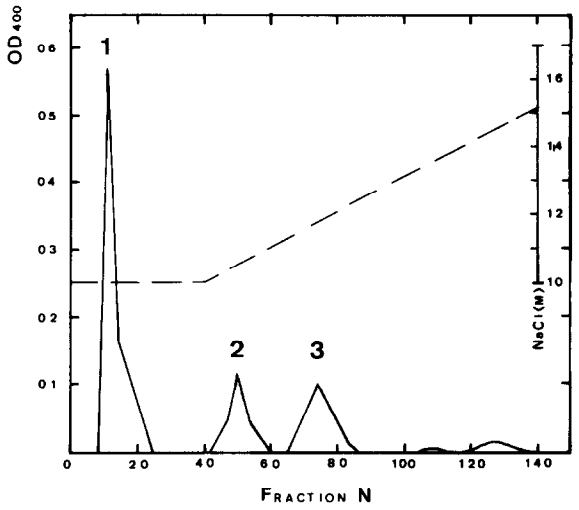

Fig.1. CM-Sephadex C25 chromatographic separation of nuclear proteins extracted from *A. ater* sperm. Peaks: 1, histone proteins; 2, proteins X and Aa6; 3, protein Aa5. (– – –) Saline gradient.

the core histone region, while protein Aa5 migrates slightly faster than histone H4. On these gels protein Aa6 resolves into at least three subcomponents. This fact might well be attributed to posttranscriptional chemical modifications (acetylation, phosphorylation, etc.) although specimen-specific variations within a given population cannot be disregarded. In SDS gels, both proteins X and Aa5 run together and slower than core histones. Protein Aa6 has the same mobility as histone H4 and appears to be homogeneous. When these proteins are run on acrylamide-Triton X-100 gels [10] their mobilities are similar to those in acetic acid-urea gels (not shown).

Determination of the M_r of proteins X, Aa5 and Aa6 has been carried out by comparing their electrophoretic mobilities with those of sperm basic proteins of known M_r [11], on acetic acid-urea gels [12,13]. Fig.3 shows the calibration curve constructed from which M_r values for proteins X, Aa5 and Aa6 of 16900, 12300 and 6700 have been respectively determined. These values are in good agreement with the data obtained from the amino acid analyses (table 1).

The amino acid compositions of proteins X, Aa5 and Aa6 are shown in table 1. The three proteins have a high content of basic residues amounting to

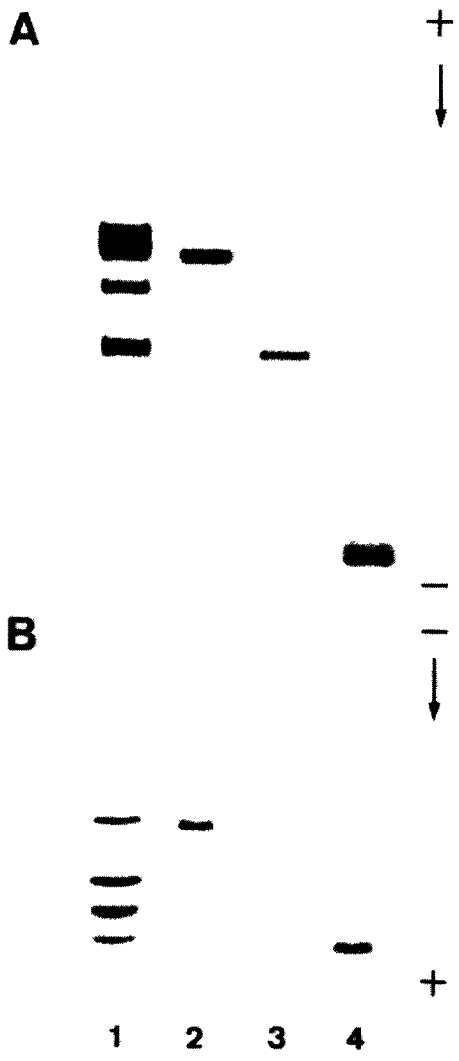

Fig.2. Polyacrylamide slab gel electrophoretic patterns in both acetic acid-urea gels (A) and acrylamide-SDS (B). Lanes: 1, starting sample from whole basic proteins from *A. ater*; 2, 3 and 4, purified proteins X, Aa5 and Aa6, respectively.

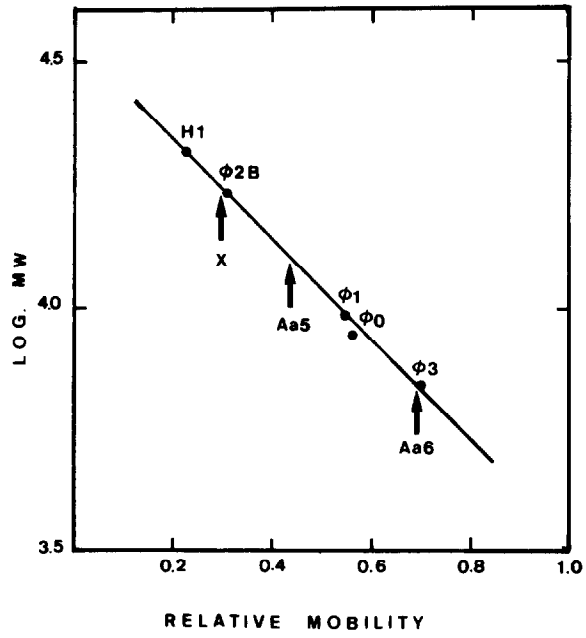

Fig.3. Correlation between electrophoretic mobility of proteins and their corresponding M_r values. Protein mobilities on 8 M urea-0.9 M acetic acid gels (15% acrylamide). Distances of migration of protein X, Aa5 and Aa6 (arrows) were measured from the origin to the midpoint of the bands.

35, 47 and 51.2% of the total residues in X, Aa5 and Aa6, respectively.

Protein X has a composition similar to that of protein φ2b from *Mytilus edulis* [1] and intermediate between somatic histones H1 and H2B. It might be considered an H2B variant provided that it has an enlarged N-terminal domain to account for the higher content of arginine, lysine and proline as found in sea urchin sperm [14,15].

Protein Aa5 has a composition rather similar to that of the highly basic protamine-like proteins described for other bivalve mollusca. It probably corresponds to protein φ1 of *M. edulis* [1], Mec1 of *M. edulus chilensis* [5], EM1 of *E. minor* [4] and protein PL2 of *C. grayanus* or *M. difficilus* [16].

Protein Aa6 is very lysine-rich and appears to be a unique type of protein, not usually found in spermatozoa of other species. It only sustains comparison to protein φ3 from *M. edulis* which contains 50% lysine.

A feature of histones is that they possess a folded, trypsin-resistant, domain. Protein X, the putative H2B, and Aa5 were digested with trypsin as outlined in section 2. Fig.4 shows the time course of digestion as visualized on acetic acid-urea gels. Histone H1 from sea urchin sperm was digested as control. Protein X gives rise to a limit-product fragment after 1–2 h digestion, as observed for the control. The appearance of a band at 10 min digestion interval, moving fasster than

Table 1

Amino acid compositions of sperm-specific proteins from *Aulacomya ater*

	X	Aa5	Aa6
Lys	22.9	26.6	41.0
His	1.3	–	–
Arg	10.8	20.4	10.1
Asp	4.8	–	–
Thr	2.7	3.4	7.8
Ser	11.2	18.7	4.7
Glu	1.0	–	1.8
Pro	8.4	10.0	9.8
Gly	6.3	2.5	1.9
Ala	15.8	16.6	21.4
Val	2.8	1.3	–
Met	1.5	–	–
Iso	3.0	–	–
Leu	5.1	–	1.7
Tyr	0.7	–	–
Phe	1.6	–	–
Lys/Arg	2.1	1.3	4.0

Values are given as molar percentage of total amino acids. Corrections have been introduced for hydrolytic losses

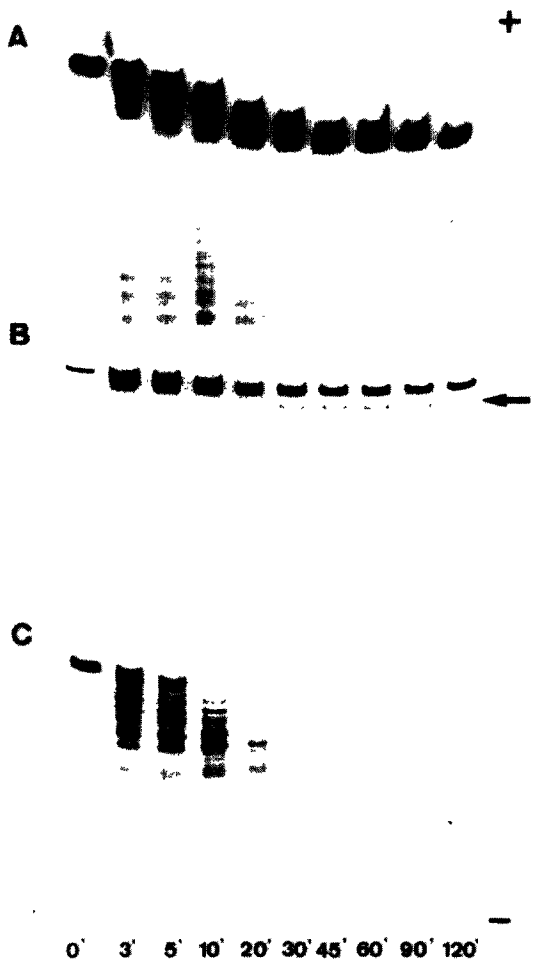

Fig.4. Acetic acid-urea gel electrophoresis of trypsin digestion products of H1 from sea urchin sperm (A), protein X (B), and protein Aa5 (C). The arrow indicates the chymotryptic product fragment.

the limit peptide, is also observed. This band has been described as being a chymotryptic product [18]. Protein Aa5 is digested totally before 60 min.

On the basis of these results, we believe that protein X can be assigned to a histone-like protein whereas both Aa5 and Aa6 can be considered protamine-like proteins. The composition of the three proteins is, in general, quite similar to that described for species within the family Mytilidae [1,3,5,17]. Therefore, these sperm-specific proteins appear to be family-specific within the class Bivalvia. This circumstance can be of value when taxonomical studies are undertaken.

Our next step is to investigate the respective primary structure of proteins in this family, since its resolution would enable us to understand better their evolutionary significance.

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